Wintering grounds, population size and evolutionary history of a cryptic passerine species from isotopic and genetic data

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SUMMARY

Cryptic species pose a particular challenge to biologists in the context of life history investigations because of the difficulty in their field discrimination. Additionally, there is normally a lag in their widespread acceptance by the scientific community once they are formally recognised. These two factors might constrain our ability to properly assess the conservation status of the different species conforming a cryptic complex. In this study, we analysed isotopic and genetic data to shed light into the still unclear wintering grounds, population size and evolutionary history of the Iberian chiffchaff (*Phylloscopus ibericus*), a species included within the Common chiffchaff (*Phylloscopus collybita*) until two decades ago due to their phenotypic similarity. We used molecular methods to identify spring-migrating *Phylloscopus* species captured in northern Iberia, and by comparing the Hydrogen isotopic ratios of their claw tips (δ^2H_c; which would reflect the signatures of their wintering grounds), we detected that δ^2H_c values of Iberian chiffchaffs were similar to Willow warblers (*Phylloscopus trochilus*; a renowned trans-Saharan migrant), and higher than Common chiffchaffs (mostly a pre-Saharan migrant). These results strongly support the idea that Iberian chiffchaffs winter in tropical Africa. We additionally reconstructed the phylogeny and evolutionary history of the Iberian chiffchaff's clade using mitochondrial and nuclear markers. Our results revealed relatively high values of nucleotide diversity (and, hence, high N_e) for the species that were greater than the values of the Common/Iberian most recent common ancestor. This suggests that the Iberian chiffchaff did not experience strong bottlenecks after diverging from the Common chiffchaff approximately one million years ago. Ultimately, our study provides another illustrative example of how isotopic and genetic analysis tools can help to enhance our understanding of avian ecology and evolution.

Keywords: COI sequences, cryptospecies, migratory bird conservation, multispecies coalescent models.

INTRODUCTION

Recent divergence, niche conservatism and morphological convergence are the main mechanisms that may lead to the existence of high phenotypic similarity between two or more genetically distinct species (Fiser et al. 2018), which are known for this reason as cryptic species (Bickford et al. 2007). The widespread use of molecular methods has revealed that cryptic species went historically unnoticed by human observers, but are actually common in most taxonomic groups (Pérez-Ponce de León & Poulin 2016), including large and iconic species such as African elephants or giraffes (Roca et al. 2001, Fennessy et al. 2016).

Cryptic diversity is very common in birds and, as in other taxonomic groups (Pérez-Ponce de León & Poulin 2016), the discovery of a cryptic species complex normally requires a re-evaluation of many fundamental questions of the ecology and evolutionary history of the newly-recognised species to reassess their conservation status independently (Struck et al. 2018). For example, these new species will typically show smaller geographic ranges and population sizes than the original pre-split species (Robuchon et al. 2019). Although this knowledge is essential for effective management and conservation of populations (Simkins et al. 2020), gathering this information normally develops very slowly for two main reasons. First, because of the inherent difficulties for the field identification and study of cryptic species; and second, because it takes some time for the existence of the new species to be widely acknowledged by the scientific community (Robuchon et al. 2019).

The avian genus *Phylloscopus* (family Phylloscopidae) includes several groups of species (*sensu* Biological Species Concept; Mayr 1942) that have been traditionally regarded as cryptic species complexes (Irwin et al. 2001, Alström et al. 2011). In the Western Palaearctic, the Iberian chiffchaff (*Phylloscopus ibericus*) constitutes a paradigmatic example. This species was considered a subspecies of the Common chiffchaff (*Phylloscopus collybita*) until the end of the 20th century, when phylogenetic analyses strongly supported its differentiation from the Common chiffchaff, although the topology of the relationships with other sister species has not been completely resolved (Helbig et al. 1996, 2001). Recent studies based on molecular markers suggest that the Iberian chiffchaff might have diverged from the Common chiffchaff during the Pleistocene, with estimations of mean divergence time dating back to 0.85 and 2.5 million years ago (Alström et al. 2018, Raković et al. 2019).

After the recognition of the taxonomic differentiation of the Iberian chiffchaff, our knowledge of its year-round distribution and population size has been constrained by the difficulty of distinguishing it morphologically (Onrubia et al. 2013a, Gordo et al. 2017) and, to some degree, acoustically from the Common chiffchaff (Helbig et al. 2001, Collinson & Melling 2008). Although the exact limits of its distribution still need to be refined, the main continuous stronghold in the breeding range of the Iberian chiffchaff would cover the

northwestern quarter of the Iberian Peninsula and the entire coastal belt of Portugal, plus some isolated breeding populations scattered in mountainous regions of southern Iberia and northern Africa (see Figure 1; BirdLife International and HBW 2019). Based on morphological studies and opportunistic sightings and ringing captures during winter, Iberian chiffchaffs are assumed to be trans-Saharan migrants that winter in tropical Africa (Svensson 2001, Pérez-Tris et al. 2003). However, the lack of ring-recovery data connecting Iberia and Africa for this species, and the limited use of molecular markers to obtain unambiguous species identifications of the captured birds challenges the reliability of these encounters during the non-breeding period (Svensson 2001). Accordingly, the winter distribution of Iberian chiffchaffs and their migratory pattern are still under discussion and require additional empirical evidence to be fully clarified (Catry et al. 2005, Clement 2015).

On a different matter, the only available information for the overall population size of the Iberian chiffchaff suggests that its numbers would range between 900,000 and 1,600,000 mature individuals (BirdLife International 2016). This estimation includes the Iberian populations and the small contingent of Iberian chiffchaffs (a few thousands) present in southwestern France. But these estimations are derived from national reports based on breeding bird survey data that was undertaken more than 15 years ago (Carrascal & Palomino 2008, Equipa Atlas 2008), when the distinction between Iberian and Common chiffchaff was still not fully-consolidated among ornithologists. This would explain why the referenced report for Spain provides large numbers of Common chiffchaffs in regions of northwestern Iberia where they are supposed to be absent (e.g. 184,000 and 116,000 individuals for Galicia and Asturias, respectively; Carrascal & Palomino 2008), which suggests that these birds would actually correspond to Iberian chiffchaffs. Therefore the official population size estimations and trends for the Iberian chiffchaff should be taken with caution (BirdLife International 2016), which calls for complementary approaches that could provide insights into its demographic history and potential conservation status.

In this study, we used information obtained from stable isotopes and genetic data to shed light on the wintering grounds, effective population size (N_e) and evolutionary history of the Iberian chiffchaff. First, we captured birds during spring migration at the northern border of Iberian chiffchaffs' breeding range, where we collected claw tips and feather samples from individuals belonging to three species of the *Phylloscopus* genus (*P. ibericus*, *P. collybita*, and Willow warbler *P. trochilus*). Feathers were used for identifying genetically Iberian chiffchaffs using the cytochrome c oxidase subunit I gene (COI), whereas the Hydrogen isotopic signatures of their claw tips (δ^2H_c) were used to infer whether they are indeed trans-Saharan migrants, by comparing their δ^2H_c values to the signatures of the other two congeneric species, whose migratory patterns and winter distributions are better known. Second, we used the COI sequences obtained in this study and genetic data from NCBI for this mitochondrial gene and

four nuclear genes (see below) to reconstruct the phylogenetic relationships among *Phylloscopus* species, and to estimate the divergence time of the Iberian/Common chiffchaffs and their effective population sizes using multispecies coalescent models (Yang 2015).

MATERIAL AND METHODS

Field and lab work for exploring the wintering grounds

We captured birds belonging to the *Phylloscopus* genus during the springs (March-April) of 2016 and 2017 in Northern Iberia (Araba province, Basque Country, Spain). These sampling sessions took place in five different woodlands of this region (see Fig. 1), where Iberian chiffchaffs are common during the breeding and migratory periods, whereas other congeneric species primarily occur on migration (Onrubia & Tellería 2013, Onrubia et al. 2013b). We collected claw tips (approximately 1 mm of each of the 8 claws: ± 0.5 mg in total) and one tail feather (one rectrix number 5) from a total of 53 spring-migrating or recently-arrived chiffchaffs, which were preliminarily identified as Common (26 birds: 14 in 2016 and 12 in 2017), Iberian (25 birds: 12 in 2016 and 13 in 2017) or uncertain (2 in 2016) according to their plumage characteristics (Onrubia et al. 2013a). We also sampled claws from 9 Willow warblers captured during their spring migration (5 in 2016 and 4 in 2017), and from 14 locally-born juvenile (Iberian) chiffchaffs (10 in 2016 and 4 in 2017) captured well into the breeding season (June-July) (Onrubia et al. 2013b). Claw tips were stored in 1,5 mL microtubes, whereas feathers were placed in paper envelopes until further analyses.

For species assignment, the calamus of the tail feather from the abovementioned 53 spring-migrating (Iberian/Common) chiffchaffs was used for DNA extraction using the Qiagen DNeasy™ Blood & Tissue Kit. Extracted DNA was used to amplify a DNA barcode region of approximately 655 base pairs of the cytochrome oxidase I (COI) gene (using Passer Forward 1 [F1] and Passer Reverse 1 [R1] primers; Lohman et al. 2009), which was sequenced to genetically assess the reliability of the Iberian/Common chiffchaff field assignations. To establish species identities according to known nucleotide sequences, we performed BLAST searches (Basic Local Alignment Search Tool; Altschul et al. 1990) using the Genbank web tool on 52 out of the 53 birds (sequencing failed for one bird assigned as Iberian chiffchaff in the field). This tool provided the COI sequences of Common (Access number: KF946814.1) and Iberian chiffchaffs (Access number: KP780413.1) respectively, as the hits with the highest percentage of similarity to our sequences. Our approach using a mitochondrial marker might have overlooked some Common/Iberian chiffchaff hybrids, but those are expected to be rare in our study setting and, if existing, would make our isotopic comparisons conservative (Helbig et

al. 2001, Bensch et al. 2002). Willow warblers were easily identified in the field (Svensson 1992), hence genetic analyses were not required for recognising this species.

A subsample (n = 40) of the abovementioned birds were used for measuring their δ^2H_c values and inferring the wintering grounds of Iberian chiffchaffs. It is important to note that Deuterium (^2H) is relatively more abundant in tropical Africa than in the Mediterranean Basin (Stefanescu et al. 2016) and δ^2H_c values in spring reflect the isotopic signatures of the areas occupied by the birds during the previous winter (Bearhop et al. 2003, Hahn et al. 2014). Isotopic measurements were obtained in the Stable Isotope Core Laboratory of Washington State University (https://www.isotopes.wsu.edu/). The 40 samples sent to this lab corresponded to four different bird groups: (1) Spring-migrating Common chiffchaffs identified according to their COI sequences (n=14; 7 in 2016 and 7 in 2017) that are primarily pre-Saharan migrants wintering in the Mediterranean Basin; (2) Spring-migrating (or recently arrived to Araba) Iberian chiffchaffs assigned according to their COI sequences (n=10; 5 in 2016 and 5 in 2017); (3) Spring-migrating Willow warblers identified according to their plumage characteristics (n=9; 5 in 2016 and 4 in 2017) representing trans-Saharan migrants that winter in tropical Africa; and (4) locally-born chiffchaffs assumed to be Iberian (n=7; 4 in 2016 and 3 in 2017), since this is by far the most common breeding chiffchaff species in the area (Gainzarain 2006, de la Hera et al. 2014). The δ^2H_c values of these juvenile chiffchaffs were used to characterize the local isotopic signal in Northern Iberia, as a control of the reliability of our comparisons, since these values are expected to be the lowest among the different groups compared according to the progressive reduction of ^2H in precipitation with latitude (Hobson et al. 2004, Stefanescu et al. 2016). We hypothesized that, if Iberian chiffchaffs are indeed trans-Saharan migrants, their δ^2H_c values would be more similar to the values shown by the Willow warbler, and higher than the Common chiffchaff.

Claw samples were first cleaned with a choroform:methanol (2:1) solution to remove impurities, and were converted into CO and H_2 with a pyrolysis elemental analyser (TC/EA Thremofinnigan, Bremen). These two gases were separated with a GC column (1.5m x ¼ ID, molecular sieve 5A, Elemental Microanalysis PN E3042) and analysed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, Termofinnigan, Bremen). Isotopic reference materials were interspersed with samples for calibration. δ^2H_c values were expressed in units per mil (‰) relative to VSMOW (Vienna Standard Mean Ocean Water) and normalized according to the values of SLAP (Standard Light Antarctic Precipitation). Note that higher values of δ^2H_c reflect a higher proportion of Deuterium (^2H) in claws.

Genetic data for estimating N_e and evolutionary history

First, we obtained 135 GenBank COI sequences (Table S1) from species belonging to the clade that includes the Iberian and Common chiffchaffs (i.e. the clade β of the Phylloscopidae family; see Alström et al. 2018). These 135 COI sequences, together with the abovementioned new 52 sequences, were used to reconstruct the phylogenetic relationships of the clade. These sequences were aligned using PRANK v.140603 (Löytynoja 2014) with default parameters (alignment is available in the Supplementary File 1). Subsequently, statistical selection of the best-fit model of nucleotide substitution was computed with jModelTest2 (Darriba et al. 2012) using the corrected Akaike information criterion for small sample size (AICc) as a selection strategy; the model TIM2+I+G showed the best fit to the data. Using this best-fit model, tree inference under maximun likelihood (ML) criterion was performed with RAxML-NG v.0.9.0 (Kozloy et al. 2019). The optimal tree was inferred using 50 starting trees (25 random and 25 parsimonious trees, respectively). Branch support for the optimal tree was calculated through the reconstruction of 1,000 bootstrap trees. The program FigTree v1.4.4 (Rambaut 2018) was used to represent the inferred optimal tree with bootstrap support.

Second, we built two datasets, one with the COI sequences longer than 600 base pairs, (hereafter mitochondrial dataset) and another with the sequences of four nuclear genes (aconitase 1 [ACO1] intron 9, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], myoglobin [MYO] intron 2, and ornithine decarboxylase [OD]; nuclear dataset) available in GenBank (see Table S1 for the list of the analysed sequences) for four *Phylloscopus* species of the clade: *P. sibilatrix*, *P. trochilus*, *P. collybita*, and *P. ibericus*. This species selection was made on the basis of maximizing the number of sequences available for *P. ibericus* to be included in the tests (files downloaded on the 16th of December 2019). Sequences from each dataset were also aligned with PRANK v.140603 (Löytynoja 2014) with default parameters (mitochondrial and nuclear alignments are available in the Supplementary File 2 and 3, respectively) and analysed as independent loci under the multispecies coalescent framework through Bayesian inference using BPP 3.4 (Rannala & Yang 2003). Each dataset was used to estimate nucleotide diversity (θ) and relative node age (τ) parameters on the fixed species phylogeny obtained during the first analytical step described above. This was done by applying the A00 analysis of the BPP program (speciesdelimitation = 0 and speciestree = 0; control file with prior and Monte Carlo Markov Chain [MCMC] iteration information is provided in the Supplementary Material; Rannala & Yang 2003, Burgess & Yang 2008, Yang 2015, Flouri et al. 2018). The BPP estimates of θ and τ were used to calculate the N_e of each species and the absolute divergence times (t) between them, respectively. This approach also allows the estimation of N_e for the most recent common ancestors (MRCA), and we considered this parameter for the Common-Iberian MRCA in order to explore the existence of population bottlenecks or expansions in the evolutionary history of the Iberian chiffchaff. The θ parameter

is directly related to N_e through the expression $\theta = 2N_e\mu g$ for the mitochondrial dataset, and $\theta = 4N_e\mu g$ for the nuclear dataset; on the other hand, the τ parameter is linked to t by the following formula $\tau = t\mu$, being μ the mutation rate and g the generation time. Information about μ for both mitochondrial and nuclear genomes is missing for the studied species. For this reason, we decided to use the μ value for the nuclear genome of the most closely-related bird species available, the Collared Flycatcher (*Ficedula albicollis*; see Smeds et al. 2016). Mutation per site per generation for this species was calculated from parent-offspring sequencing of a three-generation pedigree being 4.6×10^{-9}, which corresponds to a mutation rate of 2.3×10^{-9} mutations per site per year (Smeds et al. 2016). For the mitochondrial dataset, we applied two μ values previously used in the most recent phylogenies of the Phylloscopidae family: 2.1×10^{-8} (Alström et al. 2018) and 2.9×10^{-8} mutations per site per year (Raković et al. 2019). Estimations of N_e for each of the four *Phylloscopus* species analysed were compared to the corresponding number of mature individuals reported in the IUCN Red List of Threatened Species (BirdLife International 2016).

RESULTS

DNA-based species identifications

For the 52 chiffchaffs that yielded a COI sequence, our genetic method assigned 29 individuals as Common chiffchaffs and 23 as Iberian. Among these 29 Common chiffchaffs, 21 of them were correctly identified in the field, 6 were erroneously assigned as Iberian (21% field error rate), and the remaining birds corresponded to the two individuals with unclear external phenotype. Of the 23 Iberian chiffchaffs, 18 were successfully distinguished in the field, whereas five were wrongly determined as Common chiffchaffs (22% field error rate).

δ^2H_c comparisons

We detected significant differences in δ^2H_c values among the four *Phylloscopus* groups analysed ($F_{3,32}= 29.0$, $P < 0.001$; Fig. 2), with these values following the predicted pattern regardless of year ($F_{1,32}= 0.08$, $P = 0.781$) or group-year interactions ($F_{3,32}= 0.2$, $P = 0.897$). Thus, locally-born juvenile chiffchaffs showed the lowest δ^2H_c values, which differed significantly in the post-hoc analyses from the values exhibited by spring-migrating Common chiffchaffs ($F_{1,19}= 6.8$, $P = 0.017$), Iberian chiffchaffs ($F_{1,15}= 79.8$, $P < 0.001$) and Willow

warblers ($F_{1,14}$= 125.4, P < 0.001). As predicted according to their contrasting winter distributions, Common chiffchaffs had lower δ^2H_c values than Willow warblers ($F_{1,21}$= 33.7.4, P < 0.001). Similarly, Iberian chiffchaffs also exhibited higher δ^2H_c scores than Common chiffchaffs ($F_{1,22}$= 24.7, P < 0.001), but they did not differ from the values shown by Willow warblers ($F_{1,17}$= 0.96, P = 0.340; Fig. 2).

Phylogenetic relationships

The optimal tree based on COI gene sequences reconstructed species relationships of the clade β of the Phylloscopidae family. The tree topology with support for branches subtending species clades is represented in Figure S1. Iberian and Common chiffchaffs were recovered as sister species with strong branch support (bootstrap value of 93%). Both taxa were grouped in a well-supported clade with the Willow warbler (bootstrap value of 97%). Deeper relationships received lower support, and some species were recovered together within the same clade (*Phylloscopus schwarzi* / *Phylloscopus armandii* and *Phylloscopus proregulus* / *Phylloscopus yunnanensis*).

Divergence time and N_e

Estimations for θ and τ were supported by effective sample sizes (ESS) for MCMC, which were above 1,000 for all parameters with the exception of the nucleotide diversity of the mitochondrial dataset for the studied species quartet ($\theta_{Ps+Pt+Pc+Pi}$; see Supplementary Tables S2 and S3 for the detailed parameter estimation results). The mean divergence time between Common and Iberian chiffchaffs (τ_{Pc+Pi}) was estimated to be 0.69 million years (Ma; lower and upper Highest Posterior Density [HPD] interval 0.27 – 1.22 Ma) according to the nuclear dataset (see Figure S2A), whereas the mean of τ_{Pc+Pi} from the mitochondrial dataset (see Figure S2B) was estimated to be 1.27 Ma (HPD interval of 0.86 – 1.71 Ma) using a μ value of 2.1×10^{-8}, and 0.92 Ma (HPD interval of 0.62 – 1.24) using a μ value of 2.9×10^{-8}. N_e estimations for the Iberian chiffchaff fell within the same order of magnitude in both the mitochondrial (84,429 for $\mu = 2.1 \times 10^{-8}$; and 61,138 for $\mu = 2.9 \times 10^{-8}$) and nuclear approach (84,565; see 95% HPD intervals in Table 1). Mitochondrial N_e estimations for the Iberian Chiffchaff exceeded the N_e values for the other three extant species, but this did not occur in the nuclear dataset. Both mitochondrial and nuclear N_e estimations were slightly larger for the Iberian Chiffchaff than for the MRCA of Iberian/Common Chiffchaffs (Table 1).

DISCUSSION

By means of stable isotope analyses performed on birds whose species identity was confirmed by molecular methods, our study strongly supports that Iberian chiffchaffs winter in tropical Africa (i.e. they are trans-Saharan migrants). Likewise, a compilation of most of the genetic information shared by several *Phylloscopus* species allowed us to obtain additional data on the divergence time of the Iberian chiffchaff from the Common chiffchaff and its N_e.

Similarities between the Iberian and Common chiffchaff in their external appearance, and the less distinctive calls they emit during winter, have challenged the delimitation of the winter distribution of the former (Collinson & Melling 2008, Onrubia et al. 2013a). We cleared this hurdle using a mitochondrial genetic marker (i.e. COI sequences) that, whereas it does not provide absolute certainty in the species identification (a few rare hybrids might have been overlooked, see Methods), it ensures a higher performance in the differentiation than visual assignations. Thus, if we had relied on the field assignations based on plumage traits, we would have made more than 20 percent of identification errors, which might have biased the isotopic comparisons performed.

Originally, the idea that Iberian chiffchaffs were trans-Saharan migrants was based on three complementary lines of evidence. First, there was hardly any recovery during winter in the Iberian Peninsula of Iberian-like chiffchaffs ringed during the breeding period in that same region (Onrubia et al. 2003, Pérez-Tris et al. 2003). Second, Iberian chiffchaffs showed more pointed wings, and shorter tails than Common chiffchaffs, which is evidence of adaptation to longer migrations in the former despite their breeding in the Mediterranean area (Seebohm 1901, Salomon 1997, Pérez-Tris et al. 2003). And third, there were several sightings and ringing captures of Iberian-like chiffchaffs in the Sahel region, although for most of these records, if not all, the species identity of those individuals was not validated genetically (Svensson 2001, Zwarts et al. 2009). By making predictions on how δ^2H_c values would vary in bird claws according to the predicted differences in Hydrogen isotope ratios between tropical Africa and the Mediterranean basin (Morganti et al. 2015), our results show that Iberian chiffchaffs are trans-Saharan migrants. However, our study focuses on birds trapped in early spring on a single population near the northeastern border of the Iberian chiffchaff's breeding distribution (Fig. 1), so we cannot rule out the possibility that other conspecific populations had a different migratory behaviour, particularly in the southern part of the breeding range of the species (northern Africa). The Iberian Chiffchaffs that breed at the southernmost tip of the Iberian Peninsula (i.e. Campo de Gibraltar region) have more pointed wings than Common Chiffchaffs wintering in that same area, but their wing shape is similar to that shown by the population of Iberian Chiffchaffs analysed in this study, which would support the idea that southern populations are trans-Saharan migrants too (Pérez-Tris et al. 2003). Nevertheless, more research would be

required to clarify whether this migratory strategy is homogeneous along the entire breeding range of the Iberian chiffchaff. Our isotopic comparisons showed significant differences in the average δ^2H_c values between Common and Iberian chiffchaffs, but we also detected some degree of overlap between both species (Table S4). Thus, four Common chiffchaffs (δ^2H_c range from -55 to -13.6 ‰) had δ^2H_c scores that fell within the Iberian chiffchaff range (from -19 to 15 ‰). These individuals could belong to the contingent of Common chiffchaffs that undertake trans-Saharan migrations (Moreno-Opo et al. 2015), raising the possibility that both species could also have areas of sympatric occurrence during winter.

In recent times, an increased awareness of ornithologists along with more specific surveys has facilitated more accurate understanding of the breeding distribution of the Iberian chiffchaff (Copete 2008, BirdLife International 2016). This suggested that the baseline field data obtained in the period 2004/06 to estimate the population size of the species in Spain (see Carrascal & Palomino 2008, BirdLife International 2016) surely did not rely on criteria good enough to make a clear distinction between the recently-split Iberian and Common chiffchaffs. This would explain some obvious inconsistencies in the output of the numerical estimations: large populations of Common chiffchaffs in the northwestern part of the Iberian Peninsula, where this species is, at best, scarce; whereas not a single Iberian chiffchaff was accounted for in Andalusia (Southern Iberia), where the species is allegedly relatively common in some mountain ranges (see Carrascal & Palomino 2008). This sampling bias probably underestimated the actual population size of the Iberian chiffchaff in Spain, which is the country that contributes the most to the overall size of the species (i.e. 88% of the total population; BirdLife International 2016).

Until new field surveys that could provide a more accurate estimation of the Iberian chiffchaff numbers are made available, genetic approaches can offer an indirect way to assess the demographic history of the species. Our estimations showed a relatively high N_e for the Iberian chiffchaff,. but it is important to note that, in many cases, N_e is not a good predictor of current population size, especially for N_e estimations based on mitochondrial markers (Bazin et al. 2006). Additionally, other factors such as mating system, family group size, sex ratios, generation time and, particularly, historical fluctuations in population size can strongly affect genetic diversity and N_e estimations (Frankham 2008, Verkuil et al. 2014). For example, past population bottlenecks can have long-lasting consequences in the genetic makeup of populations (e.g. Braude & Templeton 2009). In any case, it is remarkable that the estimation of N_e for the Iberian Chiffchaff in our mitochondrial approach exceeded the values observed for the other three, much more abundant, *Phylloscopus* species (Table 1), something that did not occur for the nuclear approach. Previous studies in the Willow warbler showed that nucleotide diversity (and hence N_e) of nuclear genes tend to be higher than in the mitochondrial ones (Bensch et al. 2006, Lundberg et al. 2017). This same trend was observed in our study, with

nuclear-based N_e being one order of magnitude higher in all *Phylloscopus* species except in the Iberian chiffchaff that showed similar N_e values for both the mitochondrial and the nuclear approach. This raises the possibility that the nuclear sequences used in our study were not representative of the nuclear genome. More likely, however, these contrasting patterns would be the outcome of a study design where the individuals used for the genetic analyses are not random representations of the whole pool of genetic diversity of each species, to which the performed analyses can be sensitive (Rannala & Yang 2003, Yang 2015). Thus, for the mitochondrial dataset, most samples for the Common and Iberian chiffchaff came from individuals captured during the spring migration or early breeding at northern Spain, where only specific populations of each species could occur during this period potentially biasing the sample. Still, the Iberian chiffchaff is much better represented in sample size and geographic coverage for the mitochondrial database than the other congenerics, which would explain these relatively high mitochondrial-based N_e estimations (Table 1 and S1). A larger and more balanced sampling for all the studied species and the analysis of more genetic markers would be needed to obtain more precise estimations of the N_e. Finally, a more profound knowledge of the evolutionary history of the *Phylloscopus* species could reveal important genetic processes such as hybridizations. Hybridization of the Iberian chiffchaff with a sister species followed by genetic introgression might have also caused a high genetic diversity compared to the other species (Rheindt & Edwards 2011). In such a case, the existence of gene flow among the analysed species would make the BPP parameter estimations methodologically unsuitable (Flouri et al. 2018).

The topology and divergence times reconstructed in this study are in congruence with previous research (Alström et al. 2018, Raković et al. 2019). However, there are still some unresolved relationships, such as the position of the Canary Islands chiffchaff (*P. canariensis*), whose inclusion in future studies might shed more light into the evolutionary and demographic history of the Iberian chiffchaff. Interestingly, our preliminary results showed a larger N_e of the Iberian and Common chiffchaffs compared to the values of their MRCA (see Table 1), supporting the idea that these species did not experience strong bottlenecks during their evolutionary history. Thus, the populations of both species very likely expanded their numbers after their divergence, which would be in agreement with their relatively good conservation status to date.

Long-distance migratory passerines of small size belonging to a cryptic species complex, such as the Iberian chiffchaff, are probably the most challenging examples for ornithologists to track their movements and study different basic aspects of their biology. Ultimately, our study provides another illustrative example of how isotopic and genetic analysis tools can circumvent these problems and help to enhance our understanding of avian ecology and evolution.

Authors' contributions: IdH conceived the idea. IdH, JG, AU and MT-S designed the study. JG, AU and IdH undertook field data collection. ED and AP-R did the molecular lab work. JP-T and AP-R provided additional genetic sequences. IdH and MT-S performed the analyses. IdH wrote the manuscript with help of MT-S, JP-T, ED and AP-R. All authors commented on the manuscript and approved the final version.

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REFERENCES

Alström, P., Rheindt, F. E., Zhang, R., Zhao, M., Wang, J., Zhu, X., … Olsson, U. 2018. Complete species-level phylogeny of the leaf warbler (Aves: Phylloscopidae) radiation. – Mol. Phylogenetics Evol. 126: 141–152.

Alström, P., Saitoh, T., Williams, D., Ishiumi, I., Shigeta, Y., Ueda, K., Irestedt, M., Björklund, M. and Olsson, U. 2011. The Arctic Warbler *Phylloscopus borealis* - three anciently separated cryptic species revealed. – Ibis 153: 395–410.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. – J. Mol. Biol., 215: 403–410.

Bazin, E., Glémin, S. and Galtier, N. 2006. Population size does not influence mitochondrial genetic diversity in animals. – Science, 312: 570–572.

Bearhop, S., Furness, R. W., Hilton, G. M., Votier, S. C. and Waldron, S. 2003. A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. – Funct. Ecol. 17: 270–275.

Bensch, S., Helbig, A. J., Salomon, M. and Seibold, I. 2002. Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. – Mol. Ecol. 11: 473–481.

Bensch, S., Irwin, D. E., Irwin, J. H., Kvist, L. and Akesson, S. 2006. Conflicting patterns of mitochondrial and nuclear DNA diversity in Phylloscopus warblers. – Mol. Ecol., 15: 161–171.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K. and Das, I. 2007. Cryptic species as a window on diversity and conservation. – Trends Ecol. Evol. 22: 148–155.

BirdLife International 2016. Phylloscopus ibericus. The IUCN Red List of Threatened Species 2016: e.T22729520A87663435. http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T22729520A87663435.en. Downloaded on 15 November 2019.

BirdLife International and Handbook of the Birds of the World (2019) Bird species distribution maps of the world. Version 2019.1. Available at http://datazone.birdlife.org/species/requestdis.

Braude, S. and Templeton, A. R. 2009. Understanding the multiple meanings of 'inbreeding' and 'effective size' for genetic management of African rhinoceros populations. – African J. Ecol. 47: 546–555.

Burgess, R., and Yang, Z. 2008. Estimation of Hominoid Ancestral Population Sizes under Bayesian Coalescent Models Incorporating Mutation Rate Variation and Sequencing Errors. – Mol. Biol. Evol. 25: 1979–1994.

Carrascal, L. M. and Palomino, D. 2008. Las aves comunes reproductoras en España. Población en 2004-2006. (Seguimiento de Aves, 19). SEO/BirdLife. Madrid. 202 pp. http://www.magrama.gob.es/es/biodiversidad/temas/inventarios-nacionales/19_paseriformes_2004_2006_tcm7-218232.pdf

Catry, P., Lecoq, M., Araújo, A., Conway, G., Felgueiras, M., King, J. M. B., Rumsey, S., Salima, H. and Tenreiro, P. 2005. Differential migration of chiffchaffs *Phylloscopus collybita* and *P. ibericus* in Europe and Africa. – J. Avian Biol. 36: 184-190.

Clement, P. 2015. Iberian Chiffchaff (*Phylloscopus ibericus*). In: del Hoyo, J., Elliott, A., Sargatal, J., Christie, D.A. and de Juana, E. (eds), Handbook of the Birds of the World Alive, Lynx Edicions, Barcelona.

Collinson, J. M. and Melling, T. 2008. Identification of vagrant Iberian Chiffchaffs - Pointers, pitfalls and problem birds. – Brit. Birds 101:174-188.

Copete, J. L. 2008. Distribution and identification of Iberian Chiffchaff. – Brit. Birds 101: 378-379.

Darriba, D., Taboada, G. L., Doallo, R. and Posada, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. – Nat. methods 9: 772.

De la Hera, I., Gómez, J., Andrés, T., González-Ocio, P., Salmón, P., Salvador, M., Unanue, A., Zufiaur, F. and Onrubia, A., 2014. Inferring the migratory status of woodland birds using ringing data: the case of a constant-effort site located in the Iberian highlands. – Ardeola 61: 77–95.

Equipa Atlas. 2008. Atlas das Aves Nidificantes em Portugal (1999-2005). Instituto da Conservação da Natureza e da Biodiversidade, Sociedade Portuguesa para o Estudo das Aves, Parque Natural da Madeira e Secretaria Regional do Ambiente e do Mar. Assírio e Alvim. Lisboa

Fennessy, J., Bidon, T., Reuss, F., Kumar, V., Elkan, P., Nilsson, M. A., Vamberger, M., Fritz, U. and Janke, A. 2016. Multi-locus analyses reveal four giraffe species instead of one. – Curr. Biol. 26: 2543-2549.

Fiser, C., Robinson, C. T. and Malard, F. 2018. Cryptic species as a window into the paradigm shift of the species concept. – Mol Ecol, 27: 613–635.

Flouri, T., Jiao, X., Rannala, B. and Yang, Z. 2018. Species Tree Inference with bpp Using Genomic Sequences and the Multispecies Coalescent. – Mol. Biol. Evol. 35: 2585–2593.

Frankham, R. 2008. Effective population size/adult population size ratios in wildlife: A review. –Genet. Res. 89: 491-503.

Gainzarain, J. A. 2006. Atlas de las aves invernantes en Álava (2002-2005). Diputación Foral de Álava. Vitoria-Gasteiz.

Gordo, O., Arroyo, J. L., Rodríguez, R. and Martínez, A. 2017. Inability of biometry to discriminate Iberian and Common Chiffchaffs during the autumn migration period. – Ardeola, 64: 49-65.

Hahn, S., Dimitrov, D., Rehse, S., Yohannes, E. and Jenni, L. 2014. Avian claw morphometry and growth determine the temporal pattern of archived stable isotopes. – J. Avian Biol. 45: 202-207.

Helbig A. J., Martens J., Seibold I., Henning F., Schottler B. and Wink M. 1996. Phylogeny and species limits in the Palearctic chiffchaff *Phylloscopus collybita* complex: mitochondrial genetic differentiation and bioacoustic evidence. – Ibis 138: 650–666.

Helbig A. J., Salomon M. and Bensch S. 2001. Male-biased gene flow across an avian hybrid zone: evidence from mitochondrial and microsatellite DNA. – J. Evol. Biol. 14: 277–287.

Hobson, K. A., Bowen, G. J., Wassenaar, L. I., Ferrand, Y. and Lormee, H. 2004. Using stable hydrogen and oxygen isotope measurements of feathers to infer geographical origins of migrating European birds. – Oecologia 141: 477–488.

Irwin, D. E., Alström, P., Olsson, U. and Benowitz-Fredericks, Z. M. 2001. Cryptic species in the genus *Phylloscopus*. – Ibis 143: 233–247.

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B. and Stamatakis, A. 2019. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. – Bioinformatics, btz305.

Lohman, D. J., Prawiradilaga, D. M. and Meier, R. 2009. Improved COI barcoding primers for Southeast Asian perching birds (Aves: Passeriformes). – Mol. Ecol. Res. 9: 37–40.

Löytynoja, A. 2014. Phylogeny-aware alignment with PRANK. – Methods Mol. Biol. 1079: 155–70.

Lundberg,M., Liedvogel, M., Larson, K., Sigeman, H., Grahn, M., Wright, A., Åkesson, S. and Bensch, S. 2017. Genetic differences between willow warbler migratory phenotypes are few and cluster in large haplotype blocks. – Evol. Lett., 1: 155-168.

Mayr, E. 1942. Systematics and the origin of species. Columbia University Press. New York.

Moreno-Opo, R., Belamendía, G., Vera, P., Onrubia, A., Monteagudo, A. and de la Puente, J. 2015. Differential migration in the common chiffchaff *Phylloscopus collybita*: sub-saharan wintering grounds host more adults and females as well as birds of larger size and better physical condition. – Ardeola, 62: 237-253.

Morganti, M., Åkesson, S. and Pulido, F. 2015. Decoupling of behavioural and morphological differentiation in a partially migratory bird population. – Bird Study 62: 29-38.

Onrubia, A. and Tellería, J. L. 2013. Fenología migratoria del mosquitero Ibérico *Phylloscopus ibericus* en la Península Ibérica: una comparación con los mosquiteros común *P. collybita* y musical *P. trochilus*. Pp: 87-91 en Rodríguez, N., García, J. & Copete, J. L. (Coord.) El mosquitero ibérico. Grupo Ibérico de Anillamiento. León.

Onrubia, A., Arroyo, J. L., Andrés, T., Gómez, J., Unamuno, J. M. and Zufiaur, F. 2003. El mosquitero ibérico (Phylloscopus ibericus): identificación, biometría y apuntes sobre su migratología. – Revista de Anillamiento 12: 18-29.

Onrubia, A., Gómez, J., Andrés, T., Zufiaur, F. and Unanue, A. 2013a. Identificación en mano y determinación del sexo y edad del mosquitero ibérico *Phylloscopus ibericus*. In, N. Rodríguez, J. García-Fernández and J. L. Copete (Eds.): El mosquitero ibérico, pp. 51-60. Grupo Ibérico de Anillamiento, León.

Onrubia, A., Gómez, J., Andrés, T., Zufiaur, F., Unanue, A. and Tellería, J. L. 2013b. Migración, reproducción y muda del mosquitero ibérico *Phylloscopus ibericus* en el Norte de la Península Ibérica. Pp: 77-84 en Rodríguez, N., García, J. & Copete, J. L. (Coord.) El mosquitero ibérico. Grupo Ibérico de Anillamiento. León.

Pérez-Ponce de León, G. and Poulin, R. 2016. Taxonomic distribution of cryptic diversity among metazoans: Not so homogeneous after all. Biol. Lett. 12: 20160371.

Pérez-Tris, J., Ramírez, A. and Tellería, J. L. 2003. Are Iberian chiffchaffs *Phylloscopus* (*collybita*) *brehmii* long-distance migrants? An analysis of flight-related morphology. – Bird Study, 50: 146-152.

Raković, M., Neto, J. M., Lopes, R. J., Koblik, E. A., Fadeev, I. V., Lohman, Y. V., et al. 2019. Geographic patterns of mtDNA and Z-linked sequence variation in the Common Chiffchaff and the 'chiffchaff complex'. – PLoS ONE 14: e0210268.

Rambaut, A. 2018. FigTree version 1.4.4 https://github.com/rambaut/figtree/releases

Rannala, B. and Yang, Z. 2003. Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. – Genetics 1644: 1645–1656.

Rheindt, F. E. and Edwards, S. V. 2011. Genetic introgression: an integral but neglected component of speciation in birds. – Auk, 128: 620-632.

Robuchon, M., Faith, D. P., Julliard, R., Leroy, B., Pellens, R., Robert, A., Thévenine, C., Vérona, S., and Pavoineet, S. 2019. Species splitting increases estimates of evolutionary history at risk. – Biol. Cons. 235: 27–35.

Roca, A. L., Georgiadis, N., Pecon-Slattery, J., and O'Brien, S. J. 2001. Genetic Evidence for Two Species of Elephant in Africa. – Science 293: 1473-1477.

Salomon, M., Bried, J., Helbig, A. J. and Riofrío, J. 1997. Morphometric differentiation between male common chiffchaffs, *Phylloscopus c. collybita* Viellot, 1817, and iberian

chiffchaffs, *Phylloscopus c. brehmii* Homeyer, 1871, in a secondary contact zone (Aves: Sylviidae). – Zool. Anzeiger, 236: 25-36.

Seebohm, H. 1901. The birds of Siberia. A record of a naturalist's visits to the valleys of the Petchora and Yenesei. Cambridge University Press, Cambridge.

Simkins, A. T., Buchanan, G. M., Davies, R. G. and Donald, P. F. 2020. The implications for conservation of a major taxonomic revision of the world's birds. – Anim. Conserv. *in press*. https://doi.org/10.1111/acv.12545

Smeds, L., Qvarnström, A. and Ellegren, H. 2016. Direct estimate of the rate of germline mutation in a bird. – Genet. Res. 26: 1211–1218.

Stefanescu, C., Soto, D. X., Talavera, G., Vila, R. and Hobson, K. A. 2016. Long-distance autumn migration across the Sahara by painted lady butterflies: exploiting resource pulses in the tropical savannah. – Biol. Lett. 12: 20160561.

Struck, T. H., Feder, J. L., Bendiksby, M., Birkeland, S., Cerca, J., Gusarov, V. I., Kistenich, S., Larsson, K. H., Liow, L. H., Nowak, M. D., Stedje, B., Bachmann, L. and Dimitrov, D. 2018. Finding evolutionary processes hidden in cryptic species. – Trends Ecol. Evol. 33: 153-163.

Svensson, L. 1992. Identification guide to European Passerines. L. Svensson. Stockholm.

Svensson, L. 2001. The correct name of the Iberian Chiffchaff *Phylloscopus ibericus* Ticehurst 1937, its identification and new evidence of its winter grounds. – Bull. B. O. C. 121: 281-296.

Verkuil, Y. I., Juillet, C., Lank, D. B., Widemo, F. and Piersma, T. 2014. Genetic variation in nuclear and mitochondrial markers supports a large sex difference in lifetime reproductive skew in a lekking species. – Ecol. Evol. 4: 3626–3632.

Yang, Z. 2015. The BPP program for species tree estimation and species delimitation. – Curr. Zool. 61: 854–865.

Zwarts, L., Bijlsma, R. G., van der Kamp, J. and Wymenga, E. 2009. Living on the edge: wetlands and birds in a changing Sahel. Zeist : KNNV Publishing.

Figure Legends

Figure 1. Breeding distribution of the Iberian (dark grey) and common chiffchaffs (light grey) in the Iberian Peninsula and surrounding areas. Black areas represent regions of overlapping distributions, where both species can occur during reproduction (Source: BirdLife International and Handbook of the Birds of the World. Version 2019.1). The five woodland sites in Araba province where birds were trapped for this study are indicated using white stars.

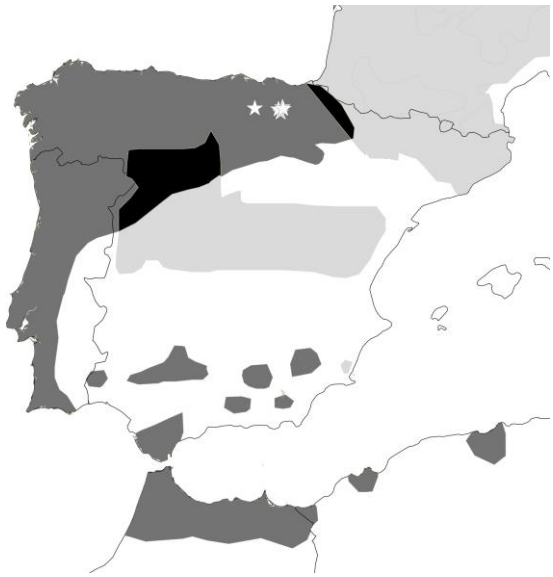

Figure 2. Variation in $\delta^{2}H_{c}$ values among the four *Phylloscopus* groups considered (Common chiffchaff *P. collybita*; Iberian chiffchaff *P. ibericus*; Willow warbler *P. trochilus*; and locally-born juvenile (Iberian) chiffchaffs) and between years (2016-2017). Graph shows means with 95% confidence intervals and sample sizes split per bird group and study year.

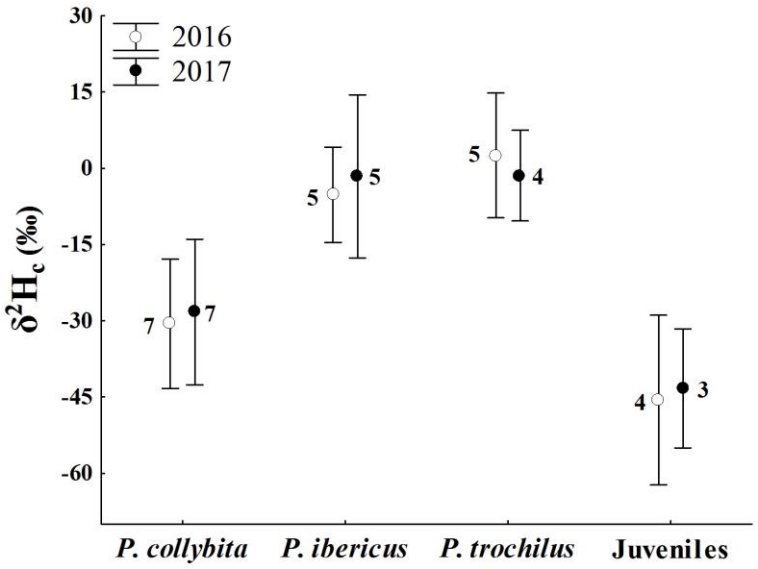

Table Legends

Table 1. Population size estimates for each of the four *Phylloscopus* species analysed in this study and the Common-Iberian chiffchaff most recent common ancestor (MRCA). Table shows estimations (mean and, in parentheses, the limits of the 95% Highest Posterior Density [HPD] intervals) of the effective population sizes (N_e) computed using both the mitochondrial (with two approaches differing in the parameter μ; see Methods) and nuclear datasets, as well as the range of mature individuals reported in the International Union for Conservation of Nature (IUCN).

	Mitochondrial DNA-based N_e estimation		Nuclear DNA-based N_e estimation	IUCN estimation of mature individuals
	$\mu = 2.1 \times 10^{-8}$ (Alström et al. 2018)	$\mu = 2.9 \times 10^{-8}$ (Raković et al. 2019)	$\mu = 2.3 \times 10^{-9}$ (Smeds et al. 2016)	
Phylloscopus sibilatrix	26,488 (6,679 – 54,762)	19,181 (4,836 – 39,655)	137,011 (52,935 – 238,859)	14,100,000 – 22,100,000
Phylloscopus trochilus	56,940 (18,702 – 103,274)	41,233 (13,543 – 74,784)	192,011 (69,402 – 348,641)	413,000,000 – 647,000,000
Phylloscopus collybita	83,095 (34,821 – 139,833)	60,172 (25,216 – 101,259)	425,815 (128,315 – 829,674)	81,900,000 – 119,000,000
Phylloscopus ibericus	84,429 (38,929 – 138,048)	61,138 (28,190 – 99,966)	84,565 (29,620 – 157,228)	926,000 – 1,530,000
MRCA *P. collybita* + *P. ibericus*	23,595 (4,179 – 58,333)	17,086 (3,026 – 42,241)	71,739 (21,685 – 143,0913)	-